Legume Futures

Legume-supported cropping systems for Europe

Work Package 3: Biodiversity Sampling Protocols

Contents:

1. Introduction

Data will be collected on the environmental impacts on biodiversity and ecosystem processes of legume-supported crop systems across the European pedo-climatic regions covered by Legume Futures. In order to generate meaningful data within the short time frame of the project these investigations will be carried out in two distinct ways. Firstly, broad comparisons between the biodiversity of novel legume and legume-supported crops and conventional crops will be made on a local basis. The biodiversity of non-crop vegetation and earthworms will be the focus of these studies. These local comparisons will be made over the two years available for sampling to provide a degree of temporal replication, and the use of as many sites as possible will generate spatial replication. The ultimate aim of this element of the study will be to determine whether there are any discernable patterns in the biodiversity of legume crops relative to conventional crops across Europe.

Secondly, the study will use more limited site-specific studies to determine the potential impacts of novel legume-supported cropping on other aspects of biodiversity and the interactions between biodiversity and ecosystem processes. Surveys of ground invertebrates at some sites will be used to assess the potential influence of legume-supported cropping on the presence of crop pests and natural predator species. Levels of soil activity and decomposition rates will be measured and related to earthworm biodiversity to give an in-depth indication of soil 'health'. Activity and decomposition rates will also be compared to data for soil N leaching and emissions as a cross-cutting exercise linking the other environmental impact assessments being carried out as part of the Legume Futures project.

Site selection:

Most sites will carry out vegetation and earthworm surveys. The small plot sizes at some sites are not ideal for these studies and the validity of data from these will be carefully assessed. However, given the presence of multiple treatment replicates and the background context of similar studies at much larger plot sizes, it is hoped that it will be possible to draw valid conclusions for all sites. Sites for the more specific questions have been selected on more stringent suitability criteria. For invertebrate sampling plots must be of sufficient size to account for the mobility of the ground invertebrates under investigation. Soil activity and decomposition will be investigated in examples of northerly and southerly sites and under different legume systems. Data on N and other environmental impacts generated by Work Package 3 will also be incorporated in analyses.

Materials required:

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Most sampling techniques require only simple and readily available materials such as 1m x 1m quadrats, small containers for pit-fall trap construction etc. These are listed in the materials section of each basic protocol. Sites will be expected to procure such items individually.

Sample verification:

Vegetation identification should be completed on site. Voucher specimens of each species encountered should be supplied to ensure the standardisation of species identification across sites and countries and to provide a data bank; see vegetation protocols for voucher specimen preparation. Earthworm samples will be sent to Ireland for identification expert under expert supervision (Olaf Schmidt, University College Dublin). Ground invertebrate samples will also be sent to Ireland for identification.

Data submission:

Data sheets will be provided to ensure the standardisation of data collection across sites. Where samples are also being submitted hard copies of data sheets should be sent in with the samples, copies of these data sheets must also be retained on site. Where only data is submitted the sheets will be submitted via email.

Timetable and submission deadlines:

To ensure the success of this relatively ambitious study it will be essential that each local data collection is carried out according to the general timetable to allow comparisons to be drawn between sites. As the geographic distribution of Legume Futures sites is so great, seasonal sampling dates should be adapted to suit the climatic conditions of each individual site. Given the limited amount of time available for the completion of the Legume Futures project, it will also be vital that samples and data are submitted very rapidly to ensure that sufficient time remains for processing and analyses.

HEALTH & SAFETY

The appropriate risk assessments must be carried out according to local/institution guidelines. The standard hazards associated with outdoor field work must be considered as well as those related to the use of potentially hazardous chemicals such as formalin and mustard oil, see 'Chemical Risk Assessment Guidelines' for further information.

2. Vegetation: Quadrat Protocol

Data on the non-crop flora of the Legume Futures field sites will be collected using simple survey methods to understand the biodiversity of legume-supported crops relative to conventional crops.

Site selection:

Most Legume Futures sites will be collecting data on vegetation biodiversity to give a broad picture of patterns in legume-supported crops across the European pedo-climatic zones.

Each site will be required to survey vegetation in a specified selection of their available plots, see 'Site-specific Requirements' for details.

Sampling Design:

Vegetation will be surveyed using standard quadrat techniques. For sites with smaller plots the required sampling density is approximately 1 quadrat per $10m^2$ to facilitate comparisons between data from the wide range of experimental sites. For sites with larger plots and field-scale experiments samples will consist of quadrats arranged along transects located within the crop.

Materials required:

- 1m x 1m fixed quadrats OR 4 canes marked to 1m
- Measuring tape/wheel/GPS to locate transects and sampling points
- Canes to mark transect start and end points in the field margin
- polybags to collect specimens
- Flower press, blotting paper, newspaper and acid-free mounting paper to prepare voucher specimens

Sampling procedure:

1. ***Locate your sampling points***

Small plots:

Randomly distribute the required number of sampling points throughout your plot. Maintain a distance >2m from the plot edge wherever possible.

Large plots/fields:

Locate two transect origins at random distances along the field margin. Permanently mark these with canes or similar markers in the field margin. If necessary, transects may be located close to crop tram-lines to minimise damage when walking through crop for sampling. To sample a transect start at the marker in the margin and walk 10m directly into the crop, stop and place the first quadrat. *If using tram-lines for access place the quadrat at least 1m into the crop to one side of the line.* Follow the 'quadrat methods' below to make your survey. Once that quadrat is finished, continue to follow a straight line through the crop locating sampling points at 10m intervals until a minimum of 10 quadrats are surveyed or the far side of the field is reached (the last quadrat should be no closer than 10m to the far field margin). Repeat this process for the second transect.

2. ***Quadrat methods***

Place a 1m x 1m quadrat around the sampling point, this may be a fixed quadrat or constructed from four 1m canes depending which is easier given the nature (height, density etc) of the crop being surveyed. Avoid trampling the vegetation within the quadrat.

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Identify all plants (including the crop(s)) present within the quadrat to species level. For each new species observed, and any which cannot be identified in the field, take a sample and store in an individual clearly labelled (*specimen number, field/plot, quadrat ID, date*) bag. Estimate the %cover of each species within the quadrat by eye. NOTE: where species overlap, total %cover can be greater than 100%.

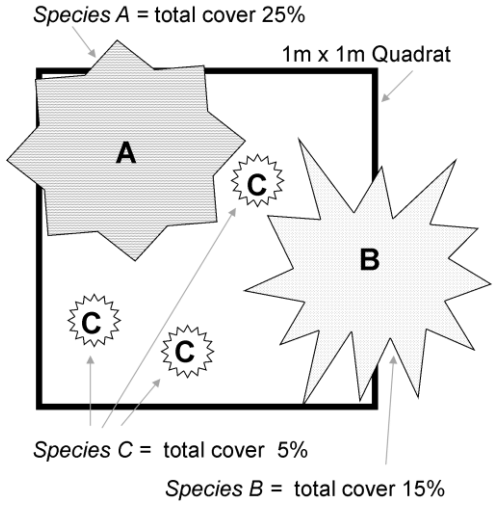

Figure 1 Example quadrat showing % cover estimates for three identified species: A, B & C.

3. *Voucher Specimens*

A voucher specimen of each identified species should be pressed and dried and sent FULLY LABELLED to *Susannah Cass, Botany Department, Trinity College, Dublin 2, IRELAND.*

- ***Herbarium specimen preparation*** [1]

Specimens should be pressed in a plant press, which consists of a wooden frame (for rigidity), corrugated cardboard ventilators (to allow air to flow through the press), blotting paper (to absorb moisture), and folded newspaper (to contain the plant material). The plant press is tightened using straps with buckles or bolts with wing nuts. The objective is to extract moisture in the shortest period of time, while preserving the morphological integrity of the plant, and to yield material that can be readily mounted on herbarium paper (an acid-free cardstock) for long-term storage.

Each specimen should consist of a stem with attached leaves and, if at all possible, flowers and/or fruits. The roots of herbaceous plants should also be included. In the case of trees, shrubs, or vines, pieces should be selected to illustrate to the greatest extent possible the overall characteristics of the plant and the range of variation in flowers, leaves, and other structures. Plants should be carefully arranged as they are placed in the press to maximise preservation of diagnostic features. Leaves, flowers, and fruits should be spread out so that they do not overlap and can be observed from different perspectives.

Each specimen should be assigned a Voucher Specimen ID consisting of an abbreviation of the site name and a unique number (see Excel data sheet - INSTRUCTIONS). The collection number should be clearly written on the outside of the newspaper containing each plant specimen. The plant press must be kept tight; this prevents shrinkage and wrinkling of the plant material and

[1] Adapted from: Marc S. Frank and Kent D. Perkins (2007) University of Florida Herbarium [Available at: http://www.flmnh.ufl.edu/herbarium/voucher.htm]

yields specimens that are easier to mount securely on herbarium paper. The pressed plants must be thoroughly dried prior to storage and mounting.

Specimens should be mounted on acid free paper and labelled using the following format:

Scientific name: genus, species, sub-species/variety/cultivar, authority

Site Details: Country, Site, Field/Plot, Quadrat ID

Plant description: describe characteristics of the plant which may be lost upon drying, such as flower/fruit colour and fragrance, leaf orientation and aroma

Collector name: Researcher(s) who collected and/or identified the specimen who can be contacted if further information is required.

Voucher Specimen ID: an abbreviation of the site name and a sequential straightforward numbering system (1, 2, 3,) for all plant species collected at your site. *Eg. The 3rd specimen from Solohead would be given the code Solo3.*

Date of collection: DD/Month/YYYY. Eg: 02 March 2011 (NOT 2/3/11 or 03/02/11)

Package mounted specimens carefully in a box or padded envelope with plenty of padding and stiff cardboard to prevent bending and post to *Susannah Cass, Botany Department, Trinity College Dublin 2, IRELAND.* Include a hard copy of the 'vegetation – species list' data sheet.

Data submission:

Enter field data into the '**LFs Biodiversity Data Sheet**' provided in the Work Package 3 shared documents area of the Sharepoint website and submit, via email, to Susannah Cass: *casss@tcd.ie*

Please submit data within two weeks of each round of sampling so that it can be compiled and submitted to Work Package 6 for modelling as soon as possible.

Timetable:

Data is required for **Spring, Summer and Autumn** weed flora. Please interpret the seasons as appropriate for your region and management practices.

Guideline examples:

- *Spring:* Data for first flush of weeds in a recently sown spring crop
- *Spring:* Data for established weeds in a maturing winter crop
- *Summer:* Data for mature weeds prior to crop harvest
- *Summer/Autumn:* Data for weeds in fallow/follow crop
- *Autumn:* Data for first flush of weeds in a recently sown winter crop

3. Earthworms: Soil Sampling Protocol

Data on the earthworm biodiversity of the Legume Futures field sites will be collected using simple survey methods to understand the biodiversity of legume-supported crops relative to conventional crops. Previous studies have shown earthworms to influence the N uptake balance of intercropped legume systems[2] and increase nitrogen uptake and crop productivity[3].

Site selection:

Most Legume Futures sites will be collecting data on earthworm biodiversity to give a broad picture of patterns in legume-supported crops across the European climate zones and soil types.

Each site will be required to sample earthworms in a specified selection of their available plots: see the 'Site-Specific Requirements'.

Sampling Design:

Earthworms will be collected using soil samples and mustard oil extraction techniques. For sites with smaller plots and multiple replicates 1-5 samples will be taken per plot. For field-scale experiments a minimum of 10 samples (in total) will be collected along the transects established for vegetation surveys. The samples should be a minimum of 10m apart and equal number should be taken from each transect.

Materials required:

- 30cm rule
- Spade – preferably with a straight, flat blade for ease of digging regular sized holes
- White or light coloured trays or sheets for sorting soil blocks on in the field
- (buckets for transporting soil to the lab if necessary – due to weather conditions etc.)
- solution of dilute mustard oil ***(see mustard oil protocol below)***
    - *Mustard oil (allyl isothiocyanate) – 2ml per 10 samples*
    - *Isopropanol (2-propanol) – low grade/cheap*
    - *Small (50ml) containers for taking concentrate into field*
    - *large water container: 20litres or more*
- Stop-clock/timer/watch to standardise 30min sorting period
- Container and fresh water to rinse mustard extracted worms
- Cool box or similar cool dark container with ice packs to transport live worms
- Paper towels for drying rinsed worms
- Watertight containers for each individual sample of worms
- 4% Formalin preservative (1:9 dilution of 40% formaldehyde in water)
- Packaging and postage for each sample set.

[2] Olaf Schmidt & James P. Curry (1999) Effects of earthworms on biomass production, nitrogen allocation and nitrogen transfer in wheat–clover intercropping model systems. *Plant and Soil* **214:** 187–198.
[3] Shujie Zhang, YingChao, ChunleiZhang, JingCheng, JunLi, NiMa (2010) Earthworms enhanced winter oilseed rape (*Brassica napus* L.) growth and nitrogen uptake. *Agriculture Ecosystems and Environment* **139(4):** 463-468.

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Aestivating earthworms indicate that the soil is too warm or dry for sampling.

When to sample:

Best results will be obtained in spring and autumn when the soil is neither frozen nor dry. Soil moisture levels must be sufficiently high for earthworm activity, as far as possible sample when soil water levels are at field capacity (soil moisture deficit = 0): when the soil contains as much water as it is able to hold without leaking[4]. If you encounter a number of worms aestivating (curled in a tight, knotted ball in the soil) the conditions are too dry for sampling and you should postpone until the next rainfall.

Sampling procedure:

locate sample point -> clear vegetation -> dig soil block -> mustard solution extraction -> 30minute soil sorting -> refill hole -> preserve worm samples in lab -> post to TCD

1. ***Mustard oil solution preparation*** *(See Chemical Risk Assessment guidelines)*

Complete a risk assessment according to the guidelines of your institution before working with allyl isothiocyanate.
Add 2ml mustard oil (allyl isothiocyanate) to 40ml Isopropanol (2-propanol, cheapest available is acceptable). This concentrate is enough to make up 20litres of mustard oil solution which will be enough for approximately 10 soil block samples. Small containers of concentrate can be made up, stored in a refrigerator and taken into the field for addition to water at the last minute.

2. ***Locate your sampling points***

In small plots randomly locate the sampling points maintaining a distance of >2m from the plot edge where possible. (On repeat sampling visits avoid re-sampling the exact position).

In large fields locate the 10 samples at random intervals >10m apart along the established transects.

3. ***Soil block sample***

Once the sampling position is selected lay out the light coloured surface (eg. tray, sheet, fertiliser sack etc) that the soil will be sorted on, also get a new collecting container ready to place any worms in. Whilst in the field the worms should be kept in collecting containers which allow air circulation, and stored in a cool dark container such as an insulated cool box/picnic box containing ice packs.

Carefully clear off any surface vegetation from the 25cm x 25cm area. Cut away the turf layer or clip vegetation with shears if necessary, and sort through this material on the tray/sheet to extract any litter dwelling worms which should be immediately placed in the collecting pot. Many worms may be burrowed in the roots of surface vegetation so pay careful attention to sorting this material.

Dig out a soil block 25cm x 25cm x 25cm and place on to the tray/sheet. Catch any worms immediately visible at the base of the hole. All of the worms collected at an individual collecting

[4] For more details on calculating soil moisture deficits see http://www.met.ie/climate/agri-meteo-data.asp .

point should be stored in the same container clearly labelled with the site, field/plot ID, sample ID and date (see LFs Biodiversity Data Sheet – INSTRUCTIONS).

4. Mustard oil extraction method

Mix up pre-prepared mustard oil concentrate (42ml) with 20litres of water. Pour approximately 1litre evenly over the base of the hole. Allow to drain into soil and repeat with a further 1litre. R**inse any earthworms which emerge in a container of fresh water immediately** (worms will die and decompose rapidly if not rinsed free of mustard solution) and continue to observe regularly whilst sorting the soil block as worms may continue to emerge after an extended period of time depending on the filtration rate of the soil. Once collecting is complete, add the rinsed worms to the collecting container with the rest of those collected from the soil sample.

5. Time limited sorting of soil sample

The 25cm x 25cm x 25cm soil blocks should be searched for earthworms of all stages: adults and juveniles. To standardise sorting methods across experimental sites, only the earthworms found by eye within 30 minutes of sifting the sample will be counted. Start the stop-clock and break up the soil block by hand over the light coloured tray/sheet. Extract worms to the collecting container as you encounter them. Observe the fine-sifted soil closely as some juvenile specimens may be very small. After 30 minutes, stop sorting. Keep each sample of earthworms in a separate container within a cool, dark box whilst in the field. Worms will die and begin to decompose if left exposed to heat and sunlight. Once sampling is complete refill the hole with the extracted soil and replace any vegetation to leave the site as tidy as possible. Move to the next sample point and repeat the previous steps.

6. Earthworm processing

Earthworms may be stored in a cool (4°C) dark container for a maximum of two days before processing. Once back in the lab rinse the earthworms in water and blot dry with paper towels. Weigh the live mass of each sample (= all worms from 1 soil block + worms from litter layer + mustard solution extracted worms) as a whole. Enter data into the '**LFs Biodiversity Data Sheet'** provided in the Work Package 3 Shared Documents area of the Sharepoint website.

Complete a risk assessment according to your institution's guidelines before working with formalin: 40% formaldehyde (see Chemical Risk Assessment guidelines). Place each sample in a watertight container with 4x more volume of formalin preservative than the volume of earthworms. CLEARLY LABEL with site, field/plot, sample ID and date. Leave for 2-3 weeks and then pour off the majority of the formalin so that worms are just covered.

Prepare samples for posting by making sure containers are tightly sealed and DO NOT LEAK, and packaged in additional sealed plastic bags. Pack containers in a sturdy box with suitable packing/cushioning material to protect containers from breaking in transit. Sample sets should be sent after each season to *Susannah Cass, Botany Department, Trinity College, Dublin 2, IRELAND. (Please label package: FRAGILE, Biological specimens in transit, HANDLE WITH CARE)*

Data submission:

Data should be compiled in the 'LFs Biodiversity Data Sheet' provided and emailed to *casss@tcd.ie*. Include copies of the completed data sheet in the package sent to Trinity College Dublin. **EARTHWORM SAMPLES MUST BE SUMBMITTED AS SOON AS POSSIBLE after each seasonal collection so that they may be identified and the data submitted for modelling.**

4. Ground invertebrates: Pit-fall Trap Protocol

Data on the ground invertebrate biodiversity of the Legume Futures field sites will be collected using pit-fall sampling to understand the biodiversity of legume-supported crops relative to conventional crops.

Site selection:

Ground invertebrate biodiversity will be investigated at a limited number of suitable Legume Futures sites. Each site will be required to set pit-fall traps in a specified selection of their available plots: see the 'Site-Specific requirements' for details.

Sampling Design:

Ground invertebrates will be sampled using pit-fall traps located along the transects established for vegetation surveys. Invertebrate diversity will be compared between legume-supported crop systems and local 'conventional' crops.

Materials required:

- A trowel or large diameter soil corer/auger to make holes in which to set traps
- Plastic cups x2 per sample – pint glasses, 500ml glasses or large yoghurt pots are all suitable (ALL TRAPS MUST BE THE SAME)
- 15cm x 15cm squares of plastic for rain cover – pale grey NOT transparent or bright coloured
- 15cm x 15cm chicken-wire type mesh covers to prevent small mammals from being trapped
- 15cm nails to support rain cover (4 per pit-fall)
- Killing fluid: car antifreeze (ethylene glycol 50% solution) + a couple of drops of unscented detergent
- Sealable containers/polybags to collect sample and trap fluid
- Paper and soft pencil for labels
- Watertight sealed specimen bags or containers to send samples by post
- 80% ethanol (preferred, or Isopropanol if ethanol unavailable) to preserve samples

Sampling procedure:

Locate sample points > set pitfall traps > return in 2-3 weeks > remove sample > re-set trap > process and preserve sample > repeat every 2-3 weeks > remove traps pre-harvest

5. *Locate your sampling points*

5 to 10 pitfall traps (in total) should be arranged along the two transects at random intervals maintaining a minimum separation of 20m and 5m from soil disturbance related to earthworm sampling. The number of pitfalls will be based on field size, see 'site-specific requirements'.

6. *Pit-fall traps*

Dig a small hole to fit the pit-fall and set the first container into the ground so that the rim is level with the soil surface. Ensure soil is filled back in around the edges of the container. Place a

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second container inside the first – it must fit closely with no gap and remain level with the soil surface. Pour the ethylene glycol solution in to 1/3 fill the inside container. Cover the trap with the mesh mammal barrier and secure it in place. Place the rain cover over the pit-fall trap and push nails into the soil to secure it at a height of 10cm above the ground.

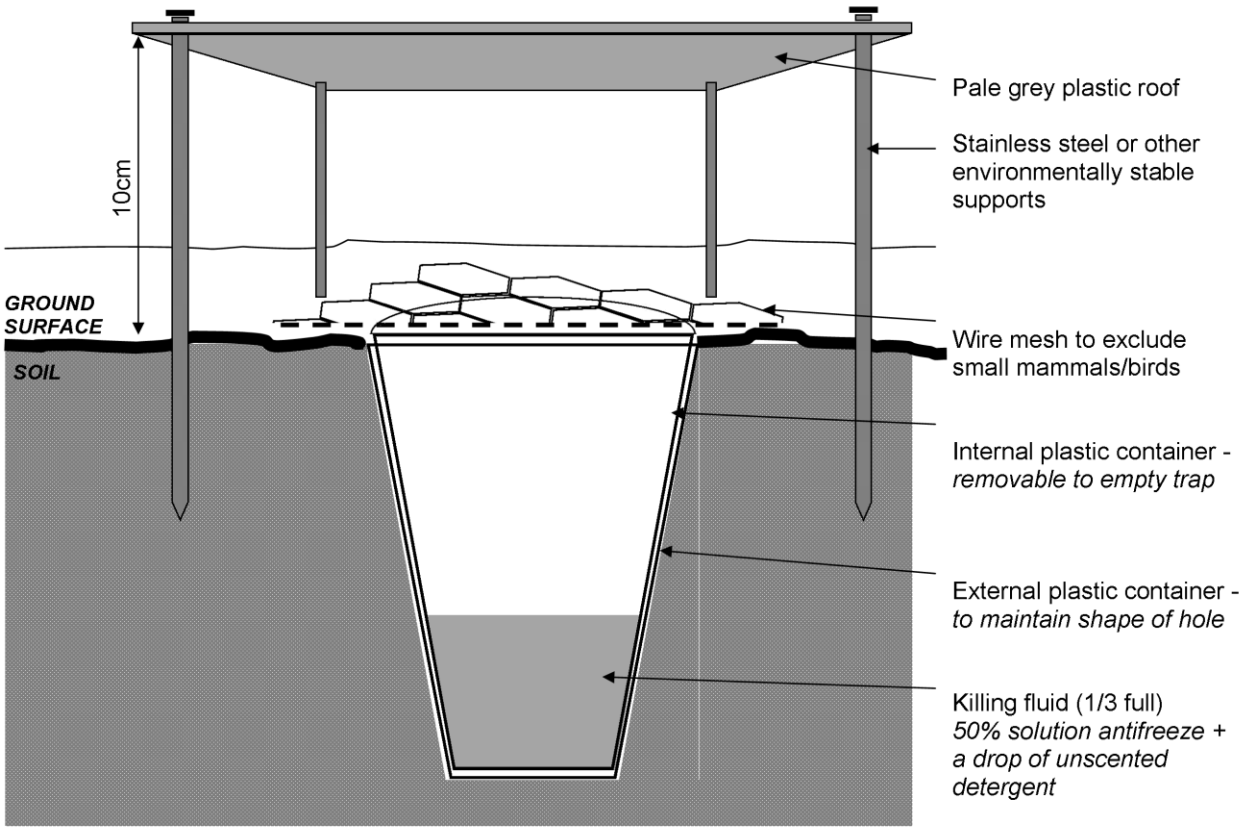

Figure 2 Pit-fall trap design and set up.

7. *Sample Collection*

After two to three weeks (empty traps more frequently in hot or wet weather), return to the pit-fall traps to take the first sample. Remove the roof and mesh cover and take out the internal container (leaving the external container to maintain the pitfall hole). Empty the sample (making sure all invertebrate specimens are collected) into a securely sealed container/polybag. Included a LABLE (site, field/plot, trap ID, date sample collected) written in soft pencil on paper INSIDE the container with the sample.

Re-set the trap with fresh antifreeze killing fluid and replace mesh and roof. Leave traps in place throughout the active Spring-Summer season, or until field is harvested/ploughed, emptying every 2-3 weeks. Remember to remove traps before harvest/ploughing!

8. *Sample processing*

Samples should be processed as soon as possible to reduce degradation. Each sample should be emptied into the aquarium net and rinsed in fresh water for several minutes to remove the killing fluid and detergent. Large debris and pieces of vegetation should be carefully removed. The sample should then be carefully rinsed out of the net with 80% ethanol into a sealable specimen bag or container. Enough alcohol should be added to at least cover the specimens and a label included on paper INSIDE the container with **site, field/plot, trap ID and date sample collected**.

EACH PIT-FALL SAMPLE MUST BE PACKAGED SEPERATELY. At the end of the trapping period, full sample sets should be strongly boxed and sent to *Susannah Cass, Botany Department, Trinity College, Dublin 2, IRELAND. (Please label package: FRAGILE, Biological specimens in transit, HANDLE WITH CARE)*

Data submission:

Use the 'LFs Biodiversity Data Sheet' provided to collect data in the field and lab. Include hard copies of completed data sheets in the package sent to Trinity College Dublin AND email a copy of the data sheet to *casss@tcd.ie*. KEEP COPIES of the data sheets to provide some information in the event that samples are lost in transit.

Timetable and submission deadlines:

Sampling should cover the **Spring-Summer** active period for ground invertebrates with traps in place continuously throughout this time to avoid missing ephemerally active species. The spring-summer season should be interpreted according to local climatic conditions and crop management calendar.